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A STUDY ON THE ULTRASTRUCTURE OF FLIGHT MUSCLE DURING THE DEVELOPMENT IN THE PUPAL STAGE OF *TENEBRIO MOLITOR* L.

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ABSTRACT

W. Y. Lee, Y. C. Lin and J. S. Su (1970) *A Study on The Ultrastructure of Flight Muscle During the Development in the Pupal Stage of* Tenebrio molitor *L.* Bull. Inst. Zool. A. S. 9(2): 83-106 The differentiation of the indirect flight muscles was studied in the prepupal and the pupal stages of *Tenebrio molitor* L. The undifferentiated cells may possibly be the precursors of the mononucleated myoblasts. The myofilament originates irregularly near the cell membranes. Several mononucleated myoblasts fuse together and then the cell membranes disappear to form the multinucleated myoblast. The myofilaments grow and aggregate to form loose myofibrils. Z-Bands begin to appear in the loose myofibrils of the third day pupa. The hexagonal arrangement of the myofilaments can be seen from the transverse section in the eighth day pupa. The formation of the striation of the sarcomere is complete at the seventh or eighth day pupae. The growth of the myofibril is resulted from the addition of myofilaments to the periphery of the fibrils. The ribosomes are seen to associate with the growth of the myofibrils. The development of the sarcosomes, and the origin and development of sarcoplasmic reticulum have also been observed and discussed.

In earlier report, the muscle differentiation, the origin, and the structure of myofibrils in the indirect flight muscle of *Drosophila melanogaster*, have been described (19). Herold (9) studied the flight muscle of the honey bee with the aid of electron microscope. Warren and Porter (21) have shown in electron micrographs that differentiation and dedifferentiation of the abdominal intersegmental muscles of *Rhodnius prolixus* are correlated with the molting cycle of the insect.

The fine structure of the indirect flight muscle in the adults of *Tenebrio molitor* L. has already been described (13). These transforming muscles during metamorphosis have been studied. As a continuation of the series in this laboratory, it is interesting to study the differentiation of the indirect flight muscles in the pupal stage with electron microscopy.

MATERIALS AND METHODS

Tenebrio molitor L. of known ages including the prepupa and the pupa was obtained from a labrotory breeding. The method to determine the stage of prepupa and pupa was described in the previous paper (14). The specimen of prepupal stage, and pupa of different ages (up to 12 days) were examined.

The indirect flight muscles from the prepupa and the pupa of different ages were fixed for 3 hours in cold 6% glutaraldehyde with 0.1M cacodylate buffer and post-fixed for 2 hours in cold 2% osmium tetroxide. All of the precedures for electron microscopic preparation were previously described [Lee *et al* (14)]. Sectioning was done on a Sorvall Porter-Blum MT-2 ultramicrotome using glass knives. Specimen were stained with 4% uranyl acetate for 2 hours before embedding or half an hour after mounting on the copper grids. The electron micrographs were taken with a Hitachi Electron Microscope Type-11A.

RESULTS

From the observation of the electron micrographs, it is suggested that the muscle fibers developed from the myoblasts which are transformed from a kind of "organelle" designated as an "undifferentiated cell" (Fig. 1a). The size of those cells is about 2.5-3.0μ in length and 1.5-2.0μ in width. One or two osmophilic lysosome (L, Fig. 1a) and the endoplasmic reticulum (Er, Fig. 1a) can be found in these cells. But the nucleus is absent.

The mononucleated myoblast are mostly of spindle or elongate in shape (Fig. 1b-c, 2, 3). Gradually it increases in size, and the nucleus (N, Fig. 1b-c, 2, 3) appears and increases in size, just as the growth of amoeba. The osmophilic lysosomes (L, Fig. 1b-c, 2, 3), the endoplasmic reticulum (Er. Fig. 1b-c, 2, 3), the mitochondria (M, Fig. 1b-c, 2, 3) and the golgi region (Gr, Fig. 1b-c, 2, 3) can be identified in the cytoplasm. In the mean time, a few young myofilaments (Mf, Fig. 1b-c, 2) appear as minute threads distributed irregularly near the cell membrane. Usually the amoeba-like pseudopodia, (Ps, Fig. 2, 3) can be seen in the myoblast. It is proposed that these pseudopodia might be the indication of either the growth or the movement of the myoblasts.

A later stage of the development of the myoblasts is shown in Figure 4. Several mononucleated myoblasts aggregate together as a multinucleated cell. The cell membranes (Cm, Fig. 4, 5) of the myoblasts are degenerated gradually, and their cytoplasm are fused. Thus, as the number of myofilaments increases, they line up in parallel in the cytoplasm. Most mitochondria become rodshaped sarcosomes. At this time, the multinucleated cell is named as the "developing muscle fibril". The ribosomes and the tracheoblast (Trb, Fig. 4) can be found in the sarcoplasm of the developing muscle fibril.

Inside the developing muscle fiber the cell membranes of the myoblast disappear, and the myofilaments (Mf, Fig. 6) as well as the sarcosome (M, Fig. 6) increase, but some nuclei are on the way of degeneration as shown in Figure 6.

The myoblasts are not only seen in the prepupal stage, but also in early pupal stage (first three days). They are fused at the periphery of developing muscle fibril (Fig. 7). Figure 8 and Figure 9b show that after the cell membranes and the nuclei of the older myoblasts have degenerated, there is only a single row of the myoblasts surrounding the developing muscle fibril. The formation of the plasma membrane (Pm, Fig. 9a) occurs after the nuclei of the myoblasts have also degenerated.

In the prepupal stage, the myofilaments (Mf, Fig. 4, 5) are lined up in parallel and scattered in the muscle fibril. After pupation, the myofilaments (Mf, Fig. 10) are aggregated but not always in parallel. The thin and the thick myofilaments (Th, Tk Fig. 10) are faintly distinguishable. Some parts of the myofilaments show darker in osmophilia staining which may be considered as the location of the Z-band in the future. The shape of the sarcosomes is elongated in the longitudinal section and round in the transverse section (M, Fig. 22), however, most of them are swollen on one end. A large number of the sarcoplasmic reticulum (Sr, Fig. 10) in vesicular form and the ribosomes (R, Fig. 10) can be found in the sarcoplasma.

In the second-day pupae, the myofilaments appear mostly in aggregation. The thin and the thick myofilaments (Th, Tk, Fig. 11) are swollen on two ends at this stage. The division of the sarcosomes (Fig. 11) presumably takes place in the middle part.

In the electron micrograph of the third day pupae, it shows that the formation of the myofibrils is well under way, and Z-bands (Z, Fig. 12) can clearly be seen. Gradual differentiation of the structure of the myofibrils can be observed from the fourth-day to the seventh-day pupae (Mfb, Fig. 13-16). Z-bands (Z) are more distinct in the fourth-day pupa (Fig. 13) and I-bands (I) appear on the fifth day. The architecture of the bands in the myofibrils arrangement as -A-I-Z-I-A- becomes recognizable in the electron micrograph of the seventh-day pupae (Fig. 16).

In the transverse section, the myofibrils (Mfb, Fig. 24) appear irregular in shapes in the fifth-day pupae. The number of the thick myofilaments (Tk, Fig. 24) varies from 7 to 56. The thin myofilaments do not arrange as orbits surrounding the thick myofilament. In the seventh day pupae, the myofibril (Mfb, Fig. 25a) is almost circular-shaped and contains ca 20 to 70 thick myofilaments (Tk, Fig. 25b). Each thick myofilament is surrounded by an uncertain number of thin myofilaments (Th, Fig. 25b).

The sarcosomes in the fourth day to the seventh-day pupae (M, Fig. 13-16) appear as long sausage-shaped ones. Some are Y-shaped in the longitudinal section. The number of the cristae is only one or two, and oriented along the long axis of the sarcosome. The phenomenon of fission can be seen in the electron micrographs of the third day pupae.

The dyads can be seen in the electron micrograph of the fourth day pupae (D, Fig. 13). Figure 14 and 15 show that the plasma membrane (Pm, Fig. 14, 15) is infolded, and the broken elements form the dyads and the T-systems. Ribosomes (R, Fig. 13-16) and microtubules (Mt, Fig. 13-16) are present.

In the eighth-day pupae, the structure of the myofibrils (Mfb, Fig. 17) is well developed. H-bands (H), I-bands (I), and Z-bands (Z) are clearly indicated in Figure 17. Most electron micrographs show that the thick myofilament (Tk, Fig. 26b) is surrounded by thin myofilaments (Th, Fig. 26b). There are ca 30 to 90 thick myofilaments in one myofibril (Mfb, Fig. 26a). The enlargement of the sarcosomes (M, Fig. 17) can be observed at this time. The cristae increase in number from 1 to 2 to 3 or more in each sarcosome.

It is obvious that the sarcosomes are divided into oval shape and short elongated shape categories in the ninth-day pupa as indicated in Figure 18 (M).

In the transverse section of the tenth-day pupae (Fig. 27a), the number of the thick myofilaments is more than one hundred in each myofibril, and the ratio of the thin and the thick myofilaments (Th, Tk, Fig. 27b) is clearly of 6 to 1, the same ratio as in adult myofibrils. The sarcosomes (M, Fig. 19) enlarge greatly in size and the cristae also increase in number. The orientation of the cristae starts in the long axis of the sarcosome (M, Fig. 17) in the eighth-day pupae, but varies in directions in the tenth and the eleventh-day pupae (M, Fig. 19, 20). Many sarcosomes keep increasing their size by swelling (M, Fig. 27a) in the tenth-day pupae. From the electron micrograph of the tenth-day pupae, a large number of the tracheoles and microtubules and the ribosomes which surround the myofibrils can be observed.

In the twelfth day pupa, the last day of the pupal stage, the muscle fibers are ready to emerge. There are more than 200 thick myofilaments (Mfb, Fig. 28a) in each myofibril, and the arrangement of the thin and the thick myofilaments (Th, Tk, Fig. 28b) appears as a hexagon. Most sarcosomes (M, Fig. 21, 28a) are well developed, although evidence of swelling and division can be seen in the electron micrographs (Fig. 21, 28a). A large number of cristae are well arranged in parallel in most sarcosomes. The amount of the ribosome (R, Fig. 21, 28a) appears more in the younger pupae than in the elder ones. The microtubules and the interruptive plasma membrane are also present in these electron micrographs.

DISCUSSION

The formation of the indirect flight muscles of *Tenebrio molitor* L. can be divided arbitrately into the following stages: 1. The transformation of undifferentiated cell into mononucleated myoblasts. 2. The aggregation and cytoplasmic fusion of myoblasts, resulting in the formation of multinucleated syncytial myofibers. 3. The formation of the myofilament. 4. The organization of myofilaments into characteristic cross-striated myofibrils. 5. The development of sarcosome and the formation of the sarcoplasmic reticulum and the T-system.

With reference to the present investigation, we should like to discuss the problem of the origin and development of fibrils and the mononucleated myoblast. Heidenhain (8) proposed that fibrils develop from submicroscopic elements. Moscona (16) described that the cytoplasmic granules containing polysaccharide and ribonucleoprotein lined up in rows and fused to form the fibrils. Some fibroblasts have been demonstrated to differentiate into myoblasts by Fischman (6) in the chick embryo and by Konigsberg (11) in his *in vitro* studies of myogenesis under the light microscope. However, no evidence has been found whether fibroblasts can transform into myoblasts; only one has reported about the origin and development of the monoucleated myoblast (19). In this investigation, many undifferentiated minute oval-shaped cells and mononucleated myoblasts varied in size are found in the cytoplasm at the prepupal stage. From the observation of the structure, it may be determined that the mononucleated myoblast develops from the minute undifferentiated cells.

The multinucleation arised by cytoplasmic fusion of the monomyoblasts has been demonstrated repeatedly in the previous studies (6, 11, 19)

The myofilaments first become visible in the electron micrographs of the young myoblast as short threads near the cell membrane. This is similar to the pattern described for *Drosophila* (19). However, in more advanced stage of *Drosophila*, the myofilaments appear to have become associated with "Z bodies" in the developing myoblasts, but in *Tenebrio molitor* L., "Z bodies" structure is never seen. The developing Z-bands appear in the third day pupa.

In the present study, it is not clear when the synthesis of thin myofilament (actin) and the thick myofilament (myosin) occur. This observation agrees with that of Hay (7), but disagrees with the conclusions of Ogawa (17). Ogawa reported that the synthesis of thin myofilament is earlier than that of the thick myofilament. In general, the number of thin myofilaments greatly exceeds that of thick myofilaments. Therefore, Fischman

(6) suggested that the immunological method used by Ogawa may not have been sensitive enough to detect small concentration of myosin in a large excess of actin. The hexagonal arrangement of the myofilaments of the developing muscles begins to appear from the fifth day pupa. This result does not agree with the observation of Fischman (6) in chick embryo, in which the hexagonal arrangement of myofilaments can be seen even in the smallest myofibrils.

The formation of striations is also a subject for discussion. Shafiq (19) found that first formed fibrils are already divided into sarcomeres by Z-bands. One popular view appears to be that the first formed myofibrils are homogeneous and acquire striation only later in the development (1, 18). In the study of *Tenebrio,* the formation of striation is similar to the pattern described by Hibbs (cited by Shafiq (19)).

Microtubules which are aligned along the developing myofibrils have been regularly observed in the developing indirect flight muscle in general and also in the *Rhodnius* abdominal intersegmental muscle (21). Porter (18) suggested the microtubules are cellular cytoskeletal elements and aid in the alignment of the myofilaments.

In the cytological criteria, it may be concluded that sarcosomes develop from mitochondria of the myoblasts. The origin of mitochondria is still unknown. Hoffman and Crigg (10) have suggested that mitochondria originated from the nucleus membrane. However, in the present investigation, there are only a few mitochondria in the young myoblast before the nucleus has been completely formed. The other suggestions are given by Lever (15) who states that the mitochondria stem from Golgi membrane and by Engfeldt *et al.* (4) who describes that precursors of mitochondria may be microbodies. However, the definite conclusion remains to be solved in the future.

De Robertis *et al.* (2) suggested that long mitochondria may be divided into smaller pieces and the fragments may grow and reorganize their structure.

In the multinucleation, the myoblast mitochondria are incorporated into the myofilament as elongated sarcosomes which line up in parallel with the forming myofibrils. Same as the observation of De Robertis in the second day pupae the sarcosomes appear to divide into oval-and rod-shaped fragments which then transform into swollen vesicles or elongated long-rod ones. Herold (9) described the vesicular sarcosomes in the developing muscles. Stegwee *et al.* (20) found the vesicular sarcosome in the regenerating flight muscle of *Leptinotarsa.* Therefore, the swollen sarcosomes not only appear in the degenerating muscles of *Tenebrio* (14), but also in the regenerating or developing muscles of *Tenebrio.* The volume of the sarcosomes and the number of their cristae are increasing while the muscle is growing, and this phenomenon is related to the different metabolic activities of the muscle in different stages. This problem has been discussed in the previous paper (13).

In the myoblast, many rough-surfaced vesicles of endoplasmic reticulum are scattered throughout its cytoplasm. This endoplasmic reticulum transforms into the sarcoplasmic reticulum in the developing muscle fibrils. The T-system is formed by invagination of the plasmic membrane or the cell membrane of the myoblast. These observations agree with that of Ezerman and Ishikawa (5) in their study in developing chick skeletal muscle in *in vitro.*

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Fig. 1a. Electron micrograph of the undifferentiated cell. These cells are mostly seen in the sections in the prepupal stage and the early pupal stage. They are distributed outside of the muscle fiber and assumed to be transformed as the myoblast. No nucleus can be seen. There are only some endoplasmic reticulum (Er) and two osmiophilic lysosomes (L). 15,000. x

Fig. 1b. The young myoblast at the higher magnification. There are many osmiophilic lysosome. (L) The mitochondria (M) and the endoplasmic reticulum (Er) scatter in the cytoplasm. It seems that the nucleus (N) begins to form. The earliest myofilament (Mf) scatter periphery near the cell membrane as the short thread. Gr, Golgi region. 50,000. x.

Fig. 1c. Electron micrograph of the young myoblast shows the nucleus (N) is grown. M, Mitochondria. Mf, Myofilament. L, Lysosomes 15,000. x.

Fig. 2. Electron micrograph shows the myoblast grown by the production of the pseudopodia (Ps). The size of nucleus (N) is increased. Er, Endoplasmic reticulum. L, Lysosome. Gr, Golgi region. M, Mitochondria. Mf, Myofilament. 15,000, x.

Fig. 3. Photograph of the myoblast. It is older than the one shown in the previous Figure. Er, Edoplasmic reticulum. L, Lysosome. N, Nucleus. Ps, Pseudopodia. 15,000. x.

Fig. 4. Electron micrograph of part of the multinuclear cell. The mononuclear cell can be still separated from the cell menbrane. Some cell menbrane (Cm) is degenerated. Cytoplasm of the neighbor myoblasts is fused together. The myofilaments (Mf) are increased and grown. Most motichondria become elongate. The lysosome are fused together as a mass. N, Nucleus. Mb, Myoblast. Trb, Tracheoblast. Pm, Plasma membrane. 10,000. x.

Fig 5. The part of the multinucleated cell at higher magnification shows that the cell membrane is broken and degenerated gradually. M, Mitochondria. L, Lysosomal mass. 22,500. x.

Fig. 6. Electron micrograph shows that the nuclei are going to degenerate (DN) after the cytoplasm of each myoblast become fused and the myofilaments (Mf) and sarcosomes (M) grow. 15,000. x.

Fig. 7. The photograph of the third day pupa. The myoblasts aggregate at the periphery of the muscle fibril. The photograph shows a young myoblast (Mb) fused to the old myoblast. 7,500. x.

Fig. 8. The photograph of the fourth day pupa shows the myoblasts line up as a single cell layer at the periphery of the muscle fibril. 7,500. x.

Fig. 9a. The photograph of the longitudinal section of the fifth day pupa. The myoblasts at the periphery of the muscle fibril begin to degnerate and to become the plasma membrane of the muscle fibril. 7,500. x.

Fig. 9b. The transverse section of the fifth day pupa shows the myoblasts surrounding the muscle fibril. A myoblast is on the muscle fibril. M, Sarcosome. Mfb, Myofibril. Pm, Plasma membrane. Bpm, Broken plasma membrane. N, Nucleus of myoblast. 7,500. x.

Fig. 10. Electron micrograph of the first day pupa. The thin and the thick myofilament (Th, Tk) separately scatter in the sarcoplasm. Some sarcosomes (M) are swollen at one end. Most of them are elongate in shape. The sarcoplasma reticulum (Sr) and the ribosomes (R) lie free in the sarcoplasm. 30, 000. x.

Fig. 11. Electron micrograph of the second day pupa. The thin and thick myofilaments (Th, Tk) aggregate together forming a loose myofibril. In two sarcosome, two portions of a sarcosome are swollen and are going to divide by the fission (F). Mt, Microtubules. R, Ribosomes. Sr, Sarcoplasm reticulum. 30,000. x.

Fig. 12. The loose myofibril is grown. The region of Z-band (Z) is distinguished by the portion of the myofilament (Mf) with more somophilus. L, Lysosome. M, Sarcosome. R, Ribosome. Sr, Sarcoplasm reticulum. 30,000. x.

Fig. 13. Electron micrograph of the fourth day pupa. The number of the myofilaments (mf) of the loose myofibrils increased. The sarcosomes (M) become a long enlargement in shape. Some are fragmented into small size sarcosomes. Dyads are formed from the broken plasma membrane. R, Ribosomes. Sr, Sarcoplasm reticulum. Z, Z-band. 30,000. x.

Fig. 14, 15. Electron micrograph of the fifth day pupa and the sixth day pupa. The formation of the myofibril (Mfb) are more advanced. The structure of the sarcomere can be recognized as the A-band (A), the I-band (I) and the Z-band (Z) in some myofibrils. R, Ribosomes. Pm, Plasma membrane. F, Fission. 30,000. x.

Fig. 16. The photograph of the seventh day pupa. A, A-band. I, I-and. Z, Z-band. Pm, Plasma membrane. 30,000. x.

Fig. 17. The photograph of the eighth day pupa. The architecture of the sarcomere has been the same as that of the mature adult. The large amount of the ribosome (R) surrounds the myofibril (Mfb) and is free in the sarcoplasm. The sarcosomes (M) are long elongate in shape. DN, the degenerating muscles. Mt, the mitotubules. Sr, the Sarcoplasmic reticulum. 30,000. x.

Fig. 18. Electron micrograph of the ninth day pupa. Most sarcosomes (M) are in the process of division. Mfb, Myofibrils. 30,000. x.

Fig. 19. Electron micrograph of the tenth day pupa. M, Sarcosomes. Mfb, Myofibrils. Tr, Tracheoles. 30,000. x.

Fig. 20. Electron micrograph of the eleventh day pupa. The diameter of the myofibrils (Mfb) increased. The sarcosome (M) appear more developed with a somewhat increase in the number of the cristae. L, Lysosomes. 30,000. x.

Fig. 21. All structures of the muscle fibrils have been well developed and ready for emergence. The myofibrils (Mfb) are well formed. The sarcosomes (M) have a large number of the cristae with parallel arrangement. The ribosomes appear more than those in the younger stages of pupae. N, Nucleus. 30,000. x.

Fig. 22. The Transverse section of the first day pupa. The sarcosomes (M) are round shape in the transverse section. The thin and the thick myofilament (Tr, Tk) can be distinguished. R, Ribosome. Sr, Sarcoplasmic reticulum. 45,000. x.

Fig. 23. The photograph of the transverse section of the third day pupa. The myofilaments (Th, Tk) aggregated to form the loose myofibril. M, Sarcosome. Mt, Microtubules. R, Ribosome. Sr, Sarcoplasmic reticulum. 45,000. x.

Fig. 24. Electron micrograph of the fifth day pupa. M, Sarcosomes. Mf, Myofilaments (Th, thin filament and Tk, thick filament). Mt, Microtubules. 45,000. x.

Fig. 25a. Electron micrograph of the seventh day pupa. The thin myofilaments (Th) surround the thick myofilaments (Tk). The A-I band junction can be seen. D, Dyads. M, Sarcosomes. Mt, Microtubules. Pm, Plasmic membrane. 45,000. x.

Fig. 25b. The higher magnification picture from myofibril at the same stage as in Fig. 25a. Showing association of the thick myofilaments (Th, Tk). M, Sarcosome. Mt, Microtubules. 80,000. x.

Fig. 26a. The photograph of the eighth day pupa. The thin and the thick myofilaments (Mfb) can be seen as a hexagonal arrangement. The number of the cristae is increased from one or two of each sarcosome to three or more each one. 45,000. x.

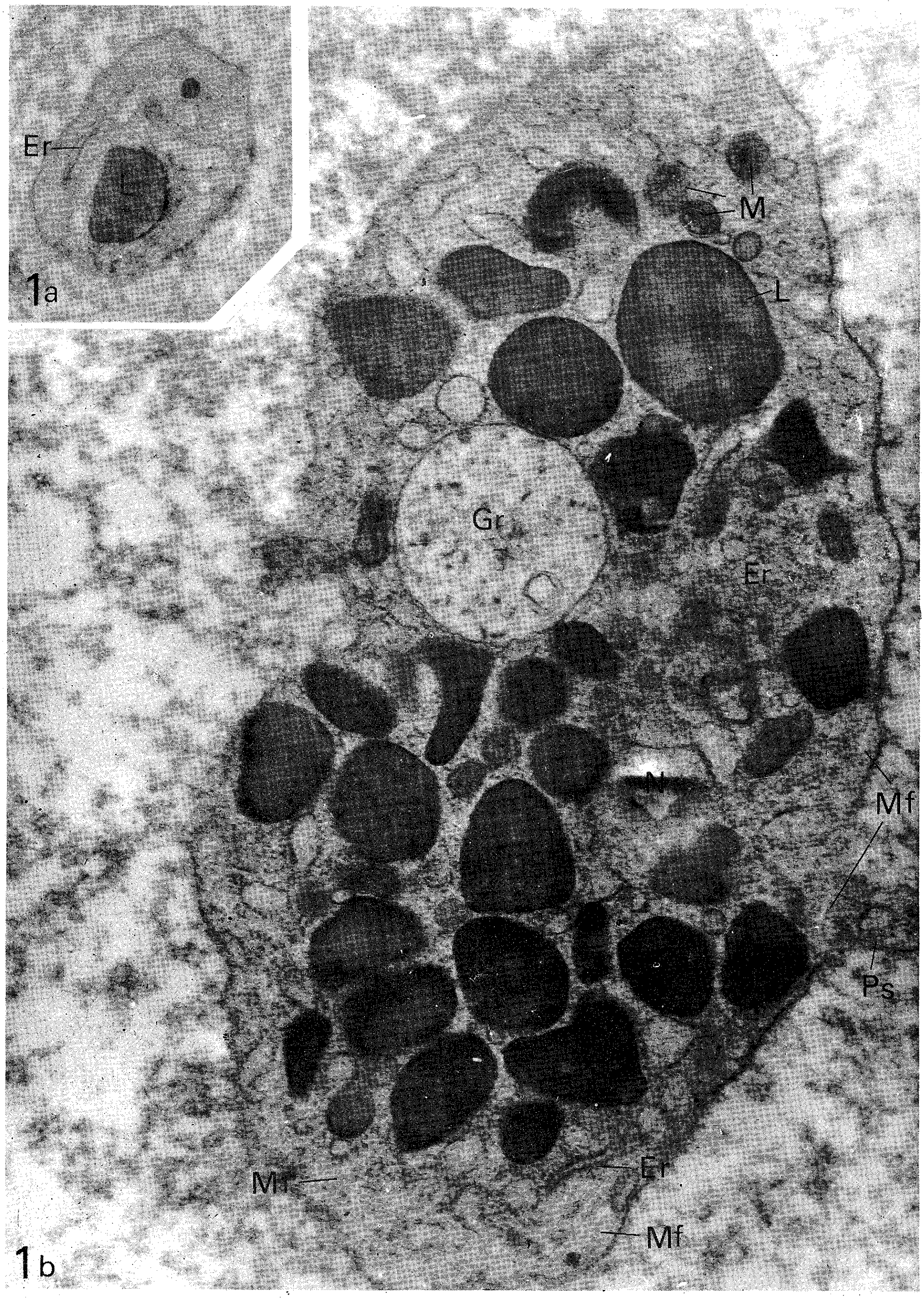
Fig. 26b. The higher magnification picture from a myofibril at the same stage of Fig. 26a. The thin and thick myofilaments (Th, Tk) take a hexagonal arrangement. R, Ribosomes. M, Sarcosome. Mt, Microtubules. 80,000. x.

Fig. 27a. The photograph of the tenth day pupa. The sarcosomes are swollen in order to increase their size. D, Dyads. Mfb, Myofibrils. Ribosomes. 45,000. x.

Fig. 27b. The higher magnification picture from a myofibril at the same stage of Fig. 27a. Mt, Microtubules. R, Ribosomes. Th, Thin myofilament. Tk, Thick myofilament 80,000. x.

Fig. 28a. The transverse section of the twelfth day pupa. M, Sarcosomes. Mfb, Myofibrils. Mt, Microtubules. Pm, Plasma membrane. R, Ribosomes. Tr, Tracheoles. 45,000. x.

Fig. 28b. The higher magnification picture from a myofibril at the same stage of Fig. 28a. M, Sarcosome. Mt, Microtubules. R, Ribosomes. Th, Thin myofilament. Tk, Thick myofilament. 80,000. x.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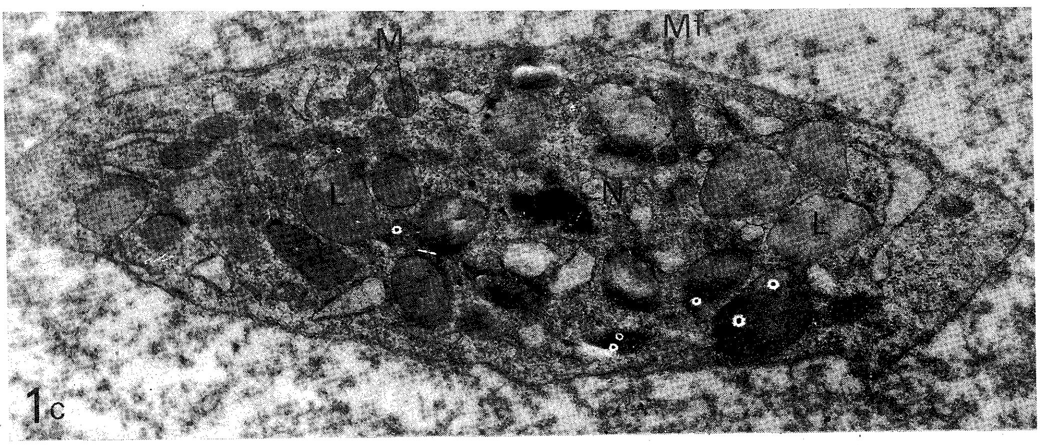
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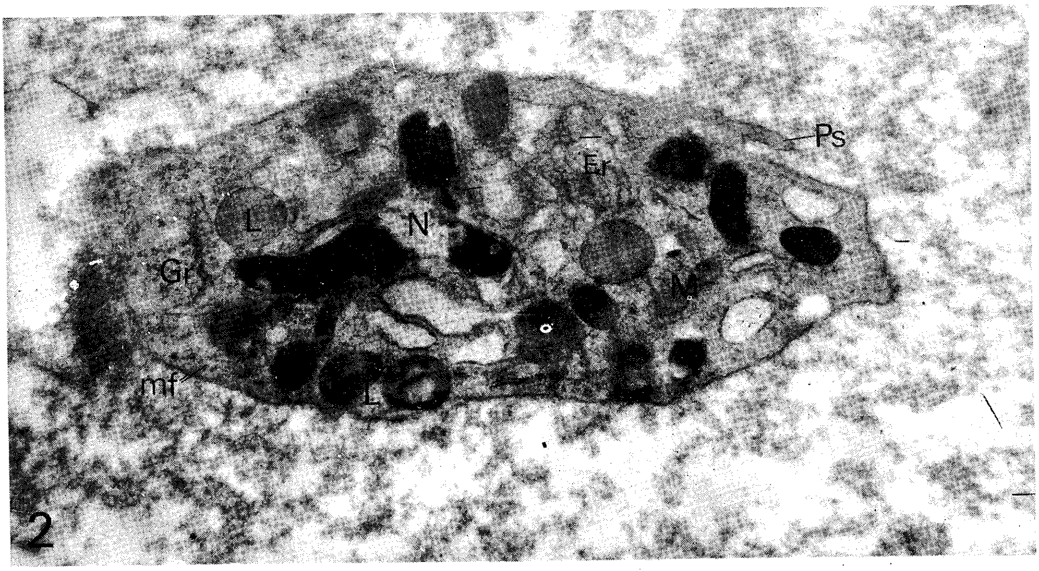
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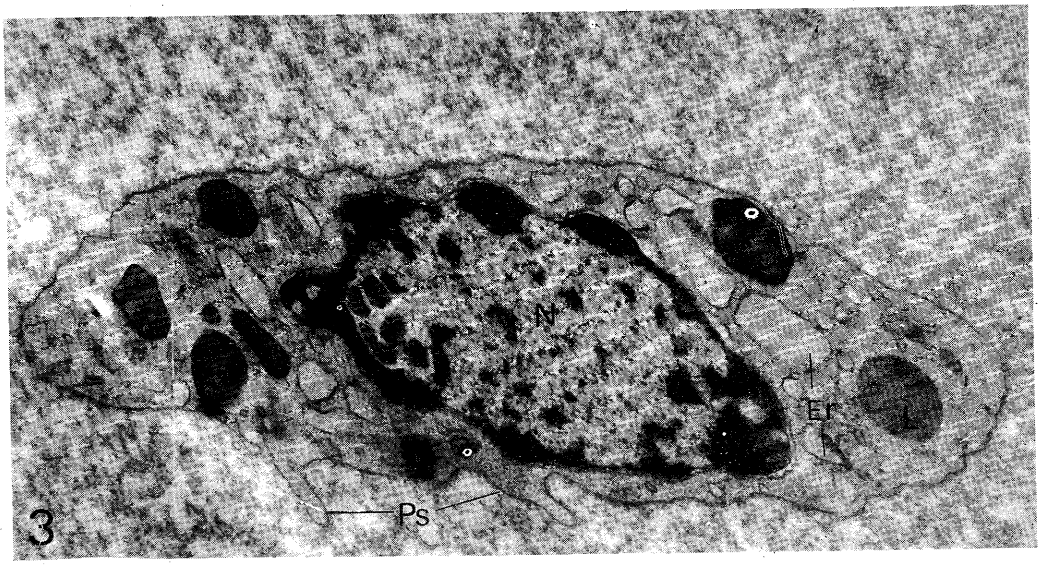
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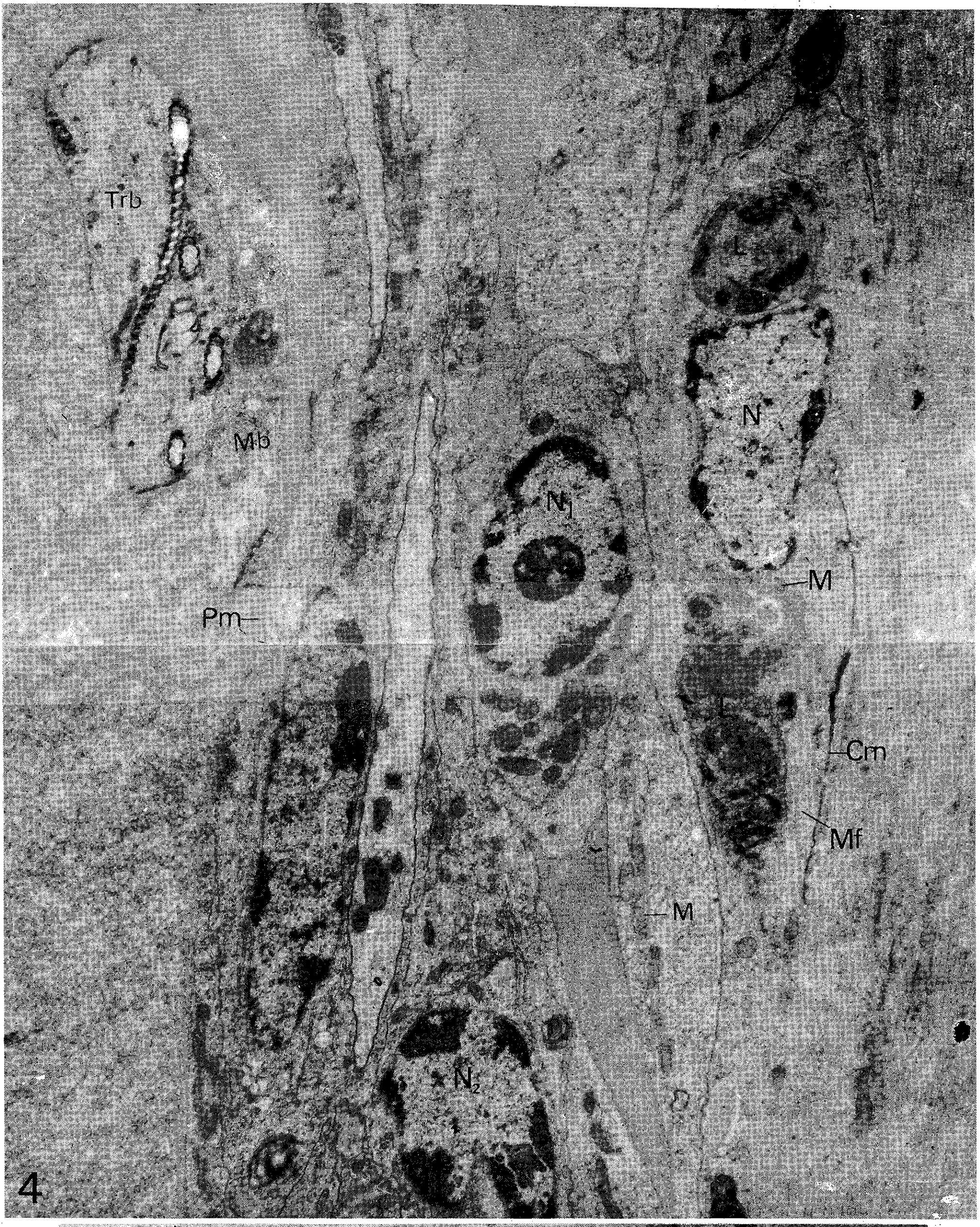

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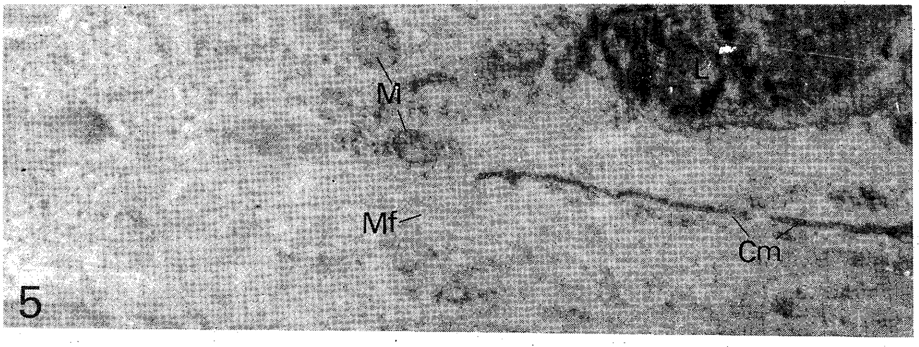

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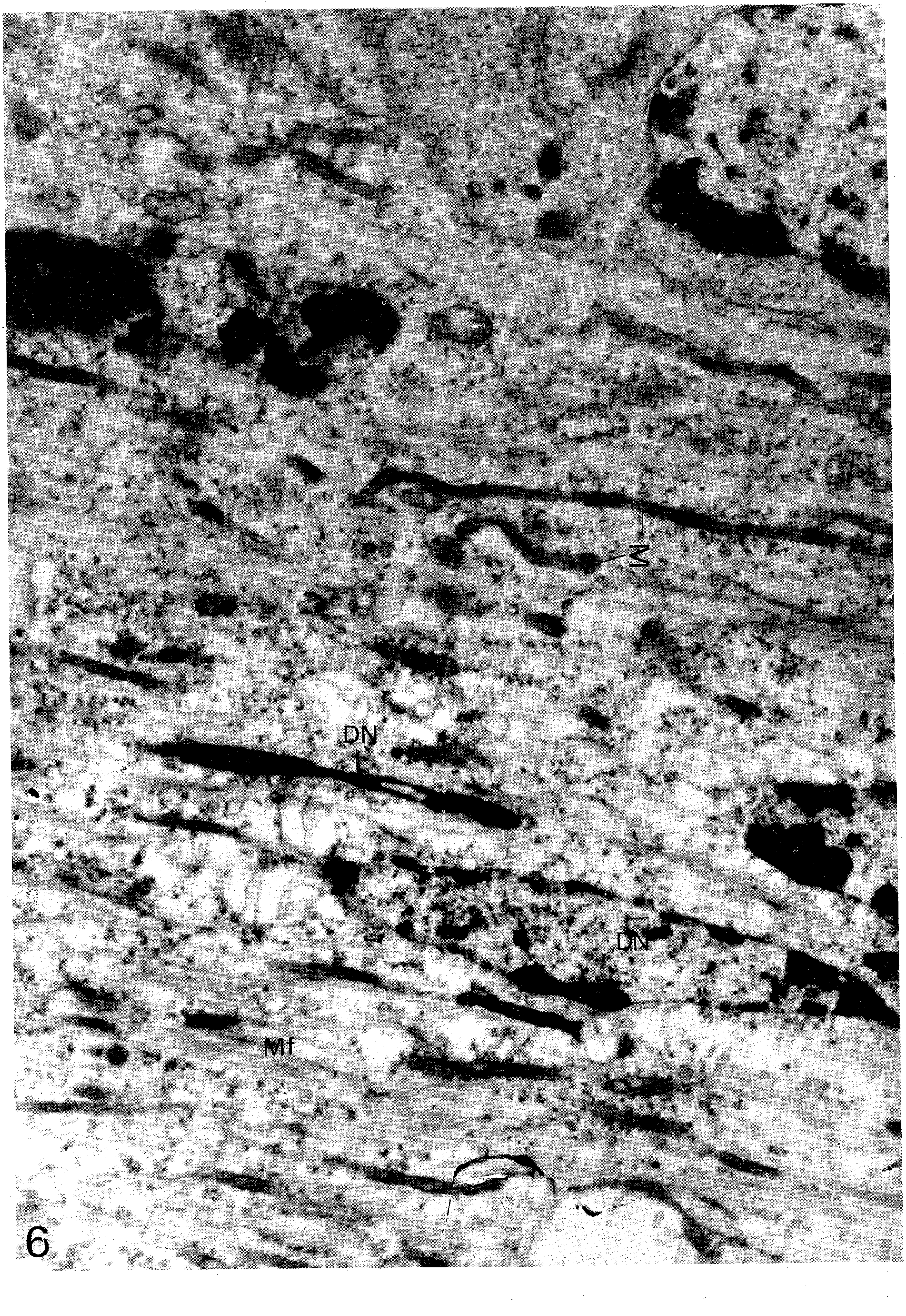
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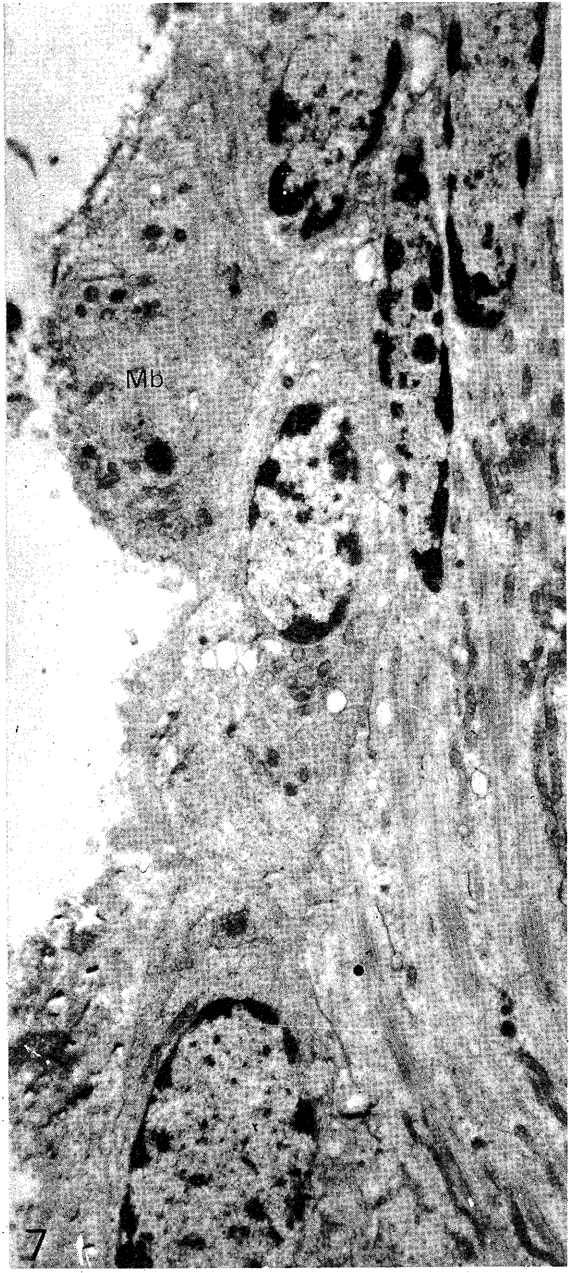
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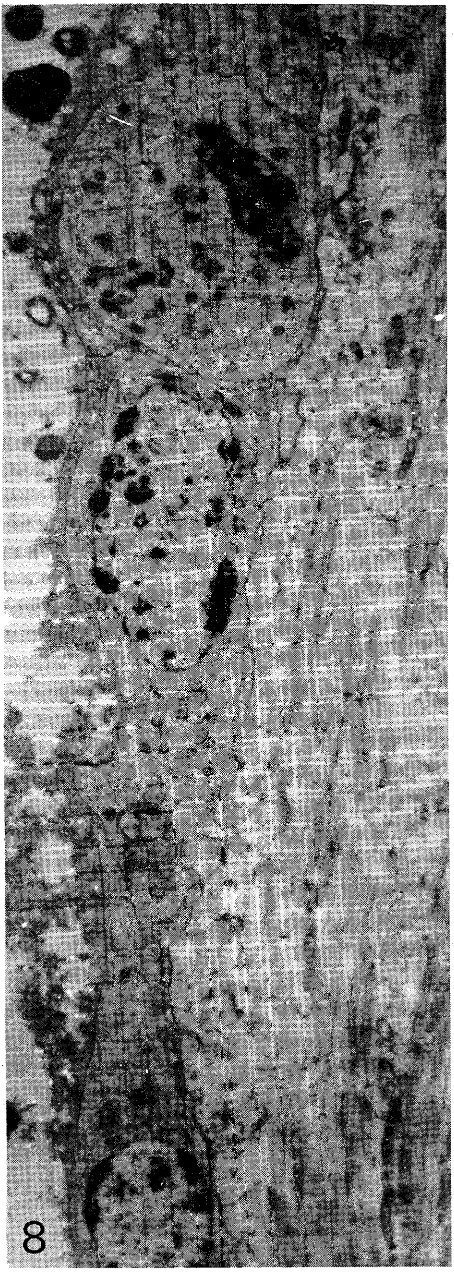
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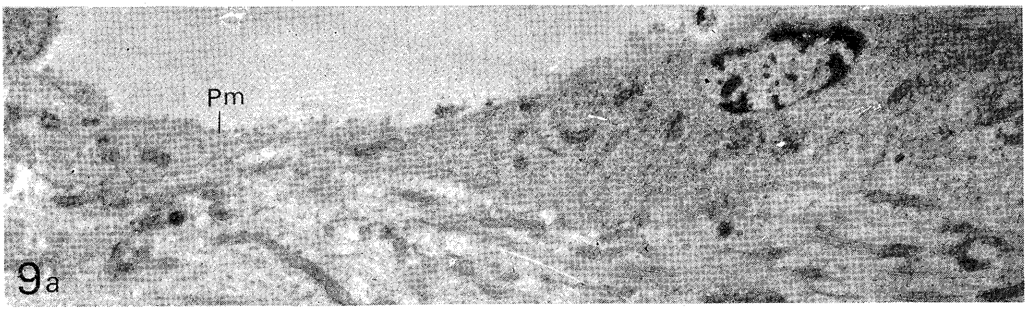
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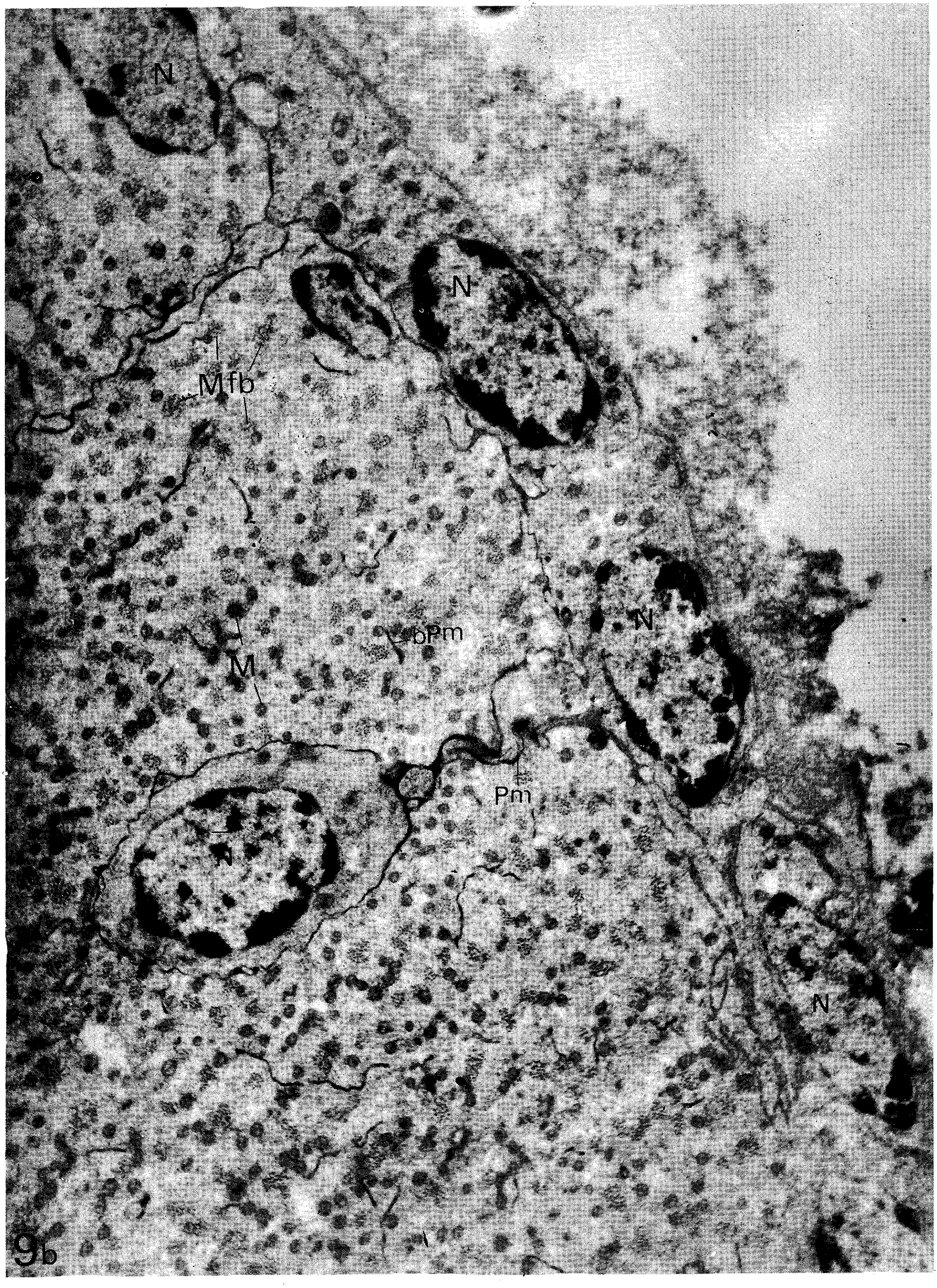

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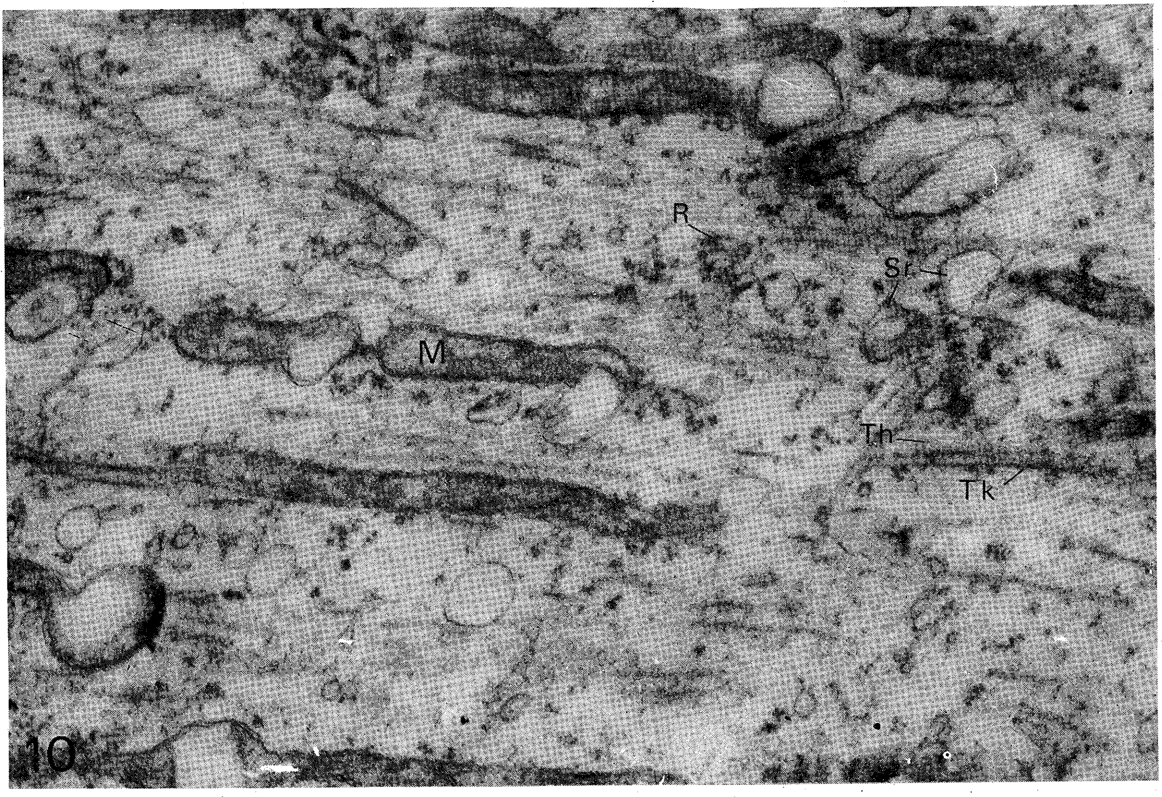

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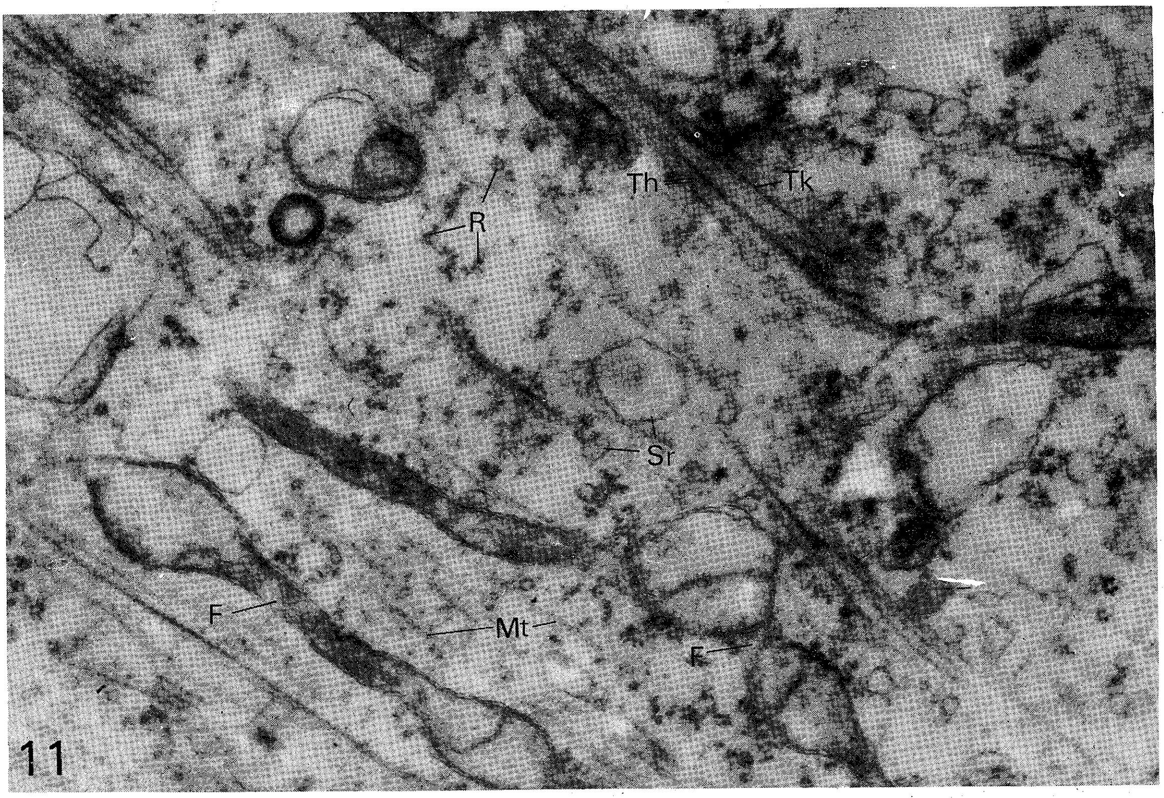

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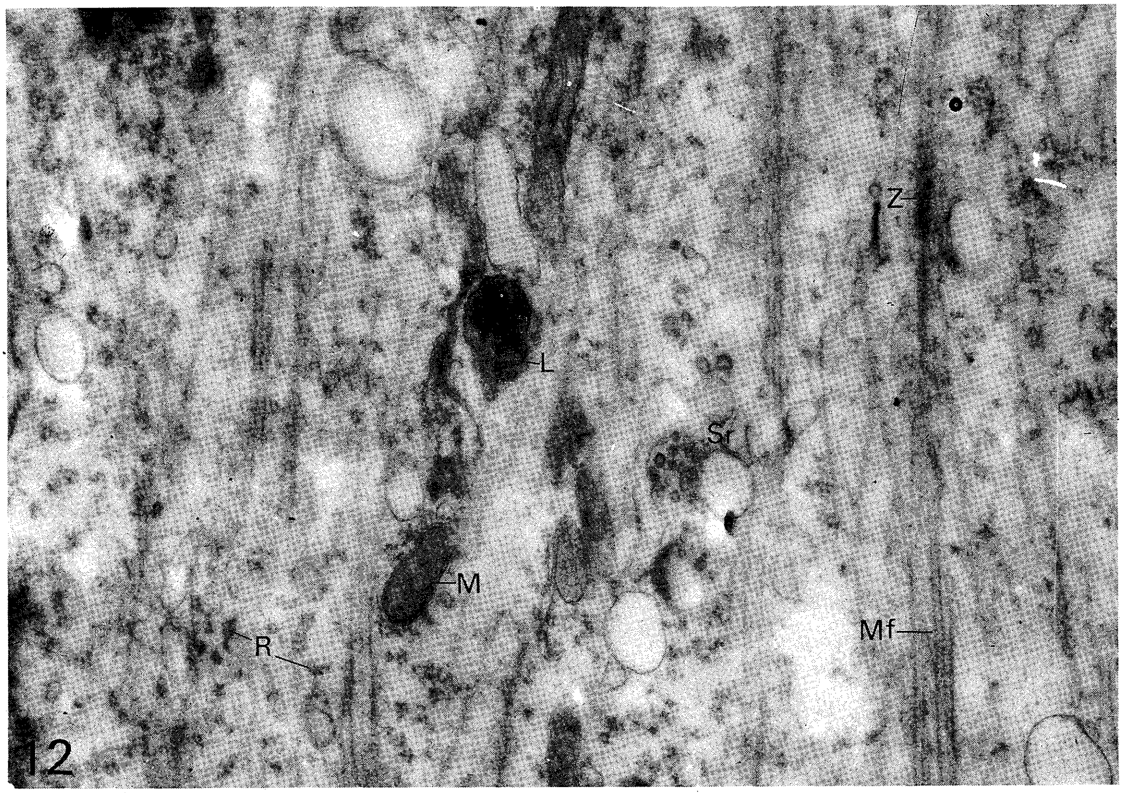
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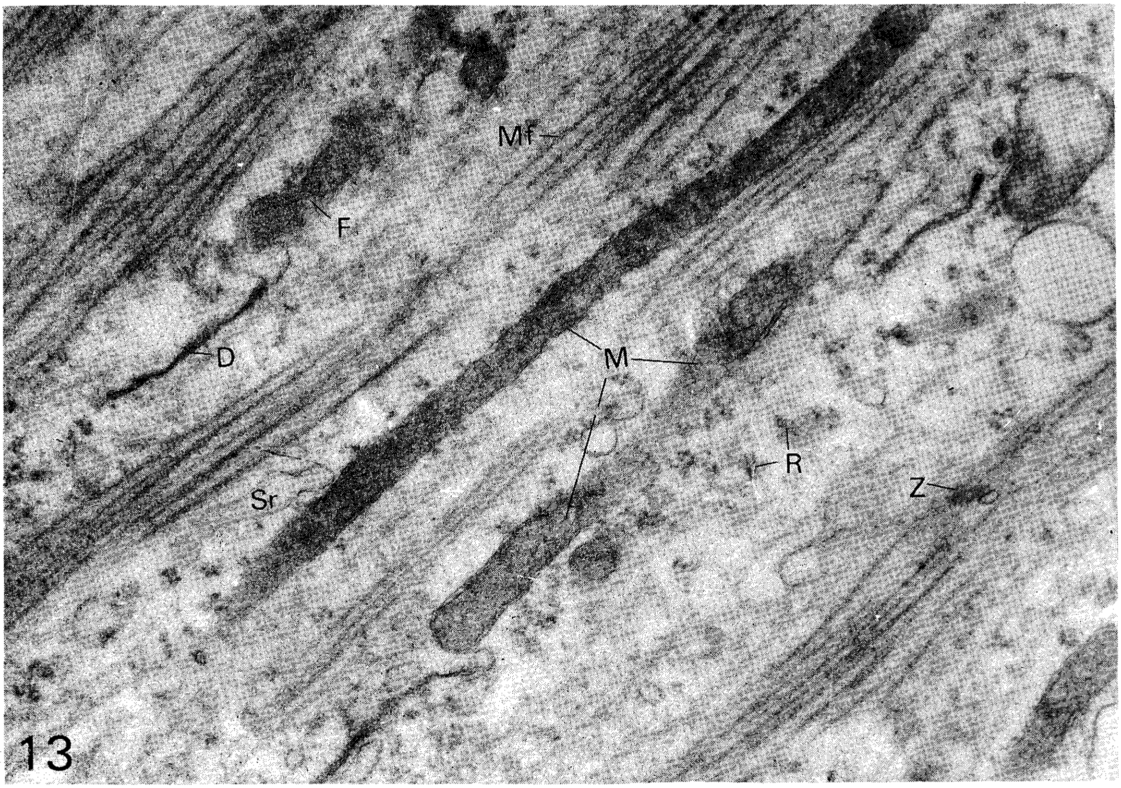
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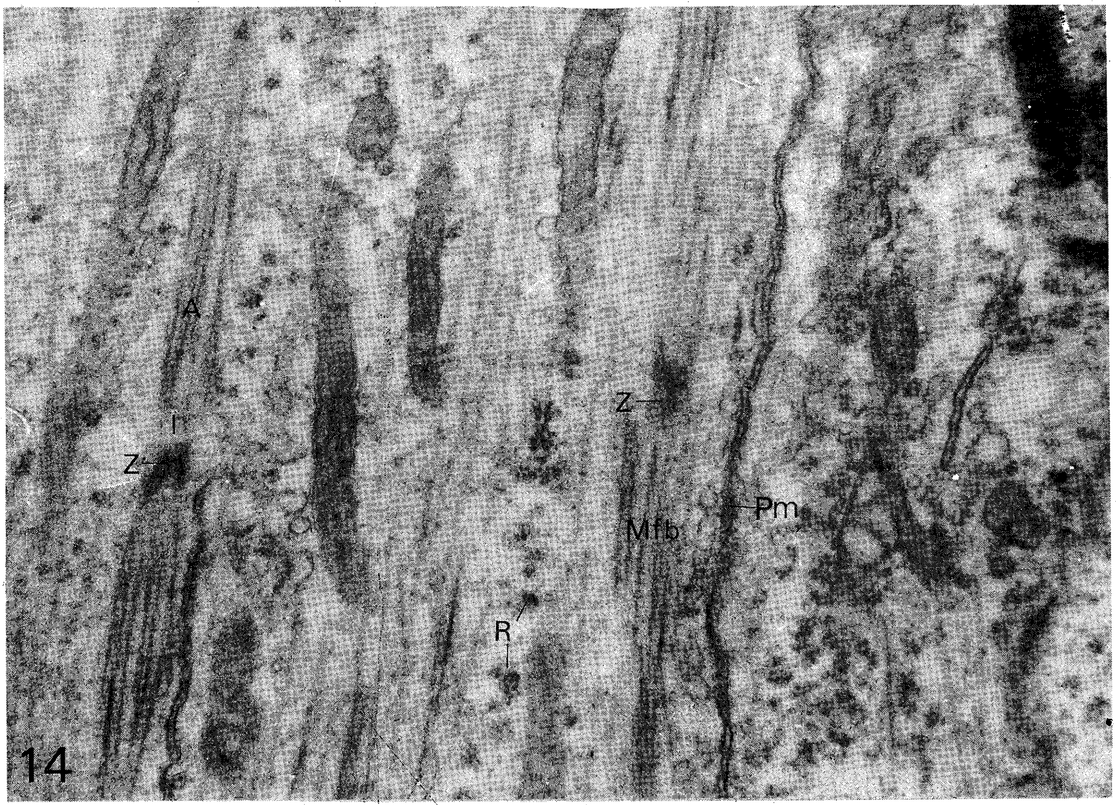

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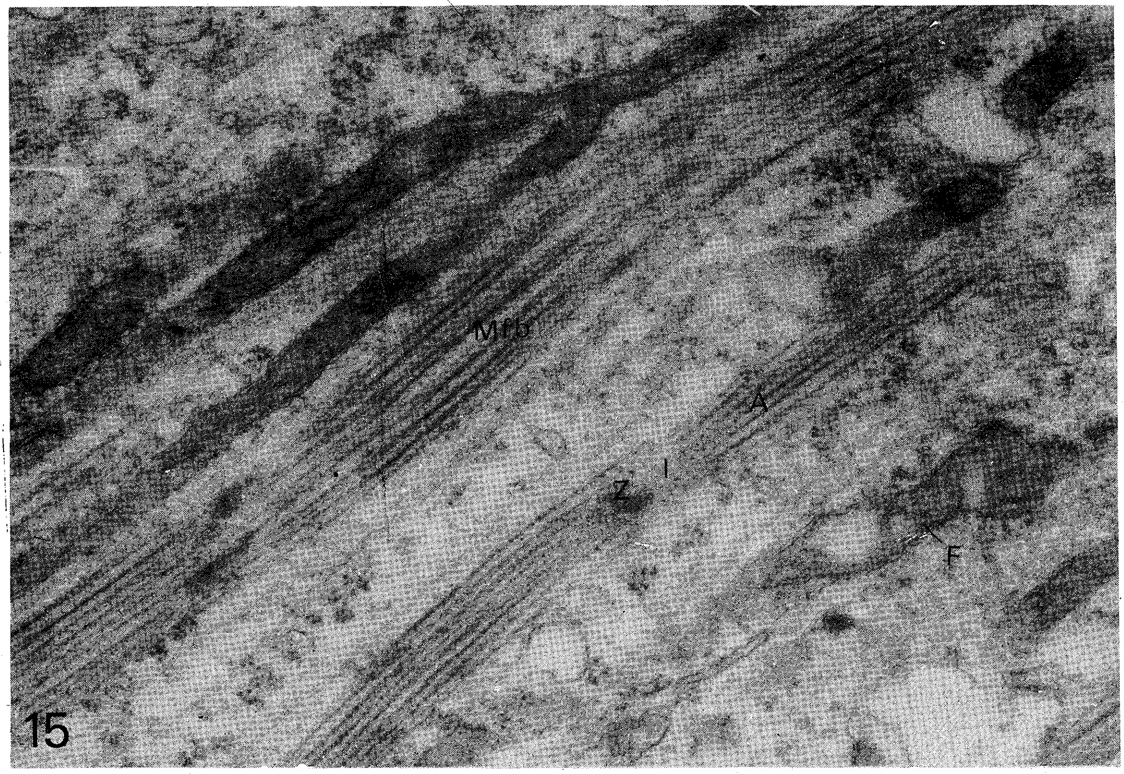

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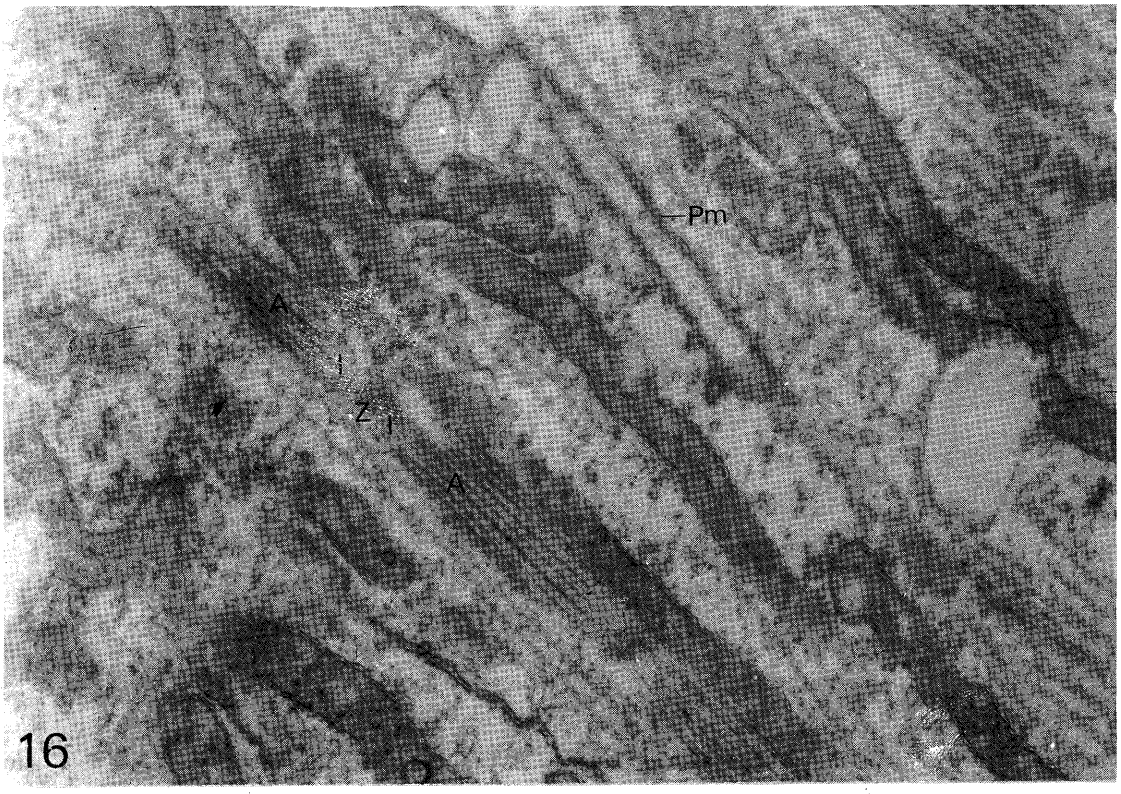

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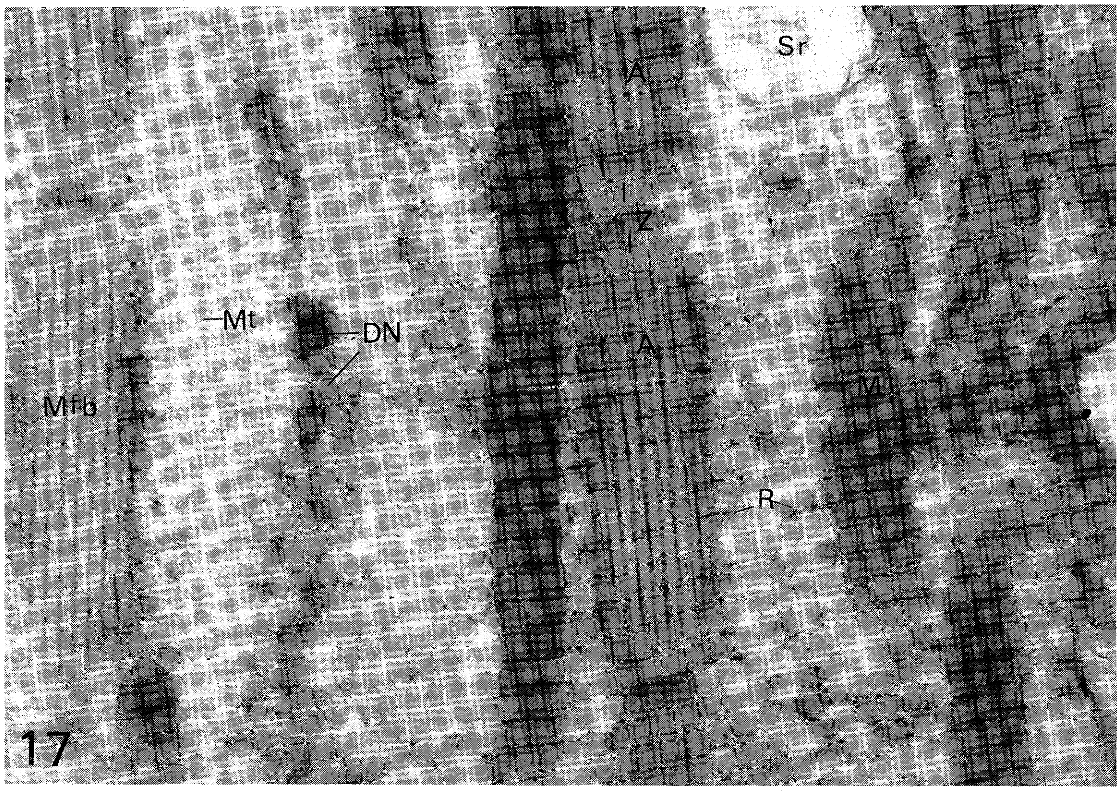

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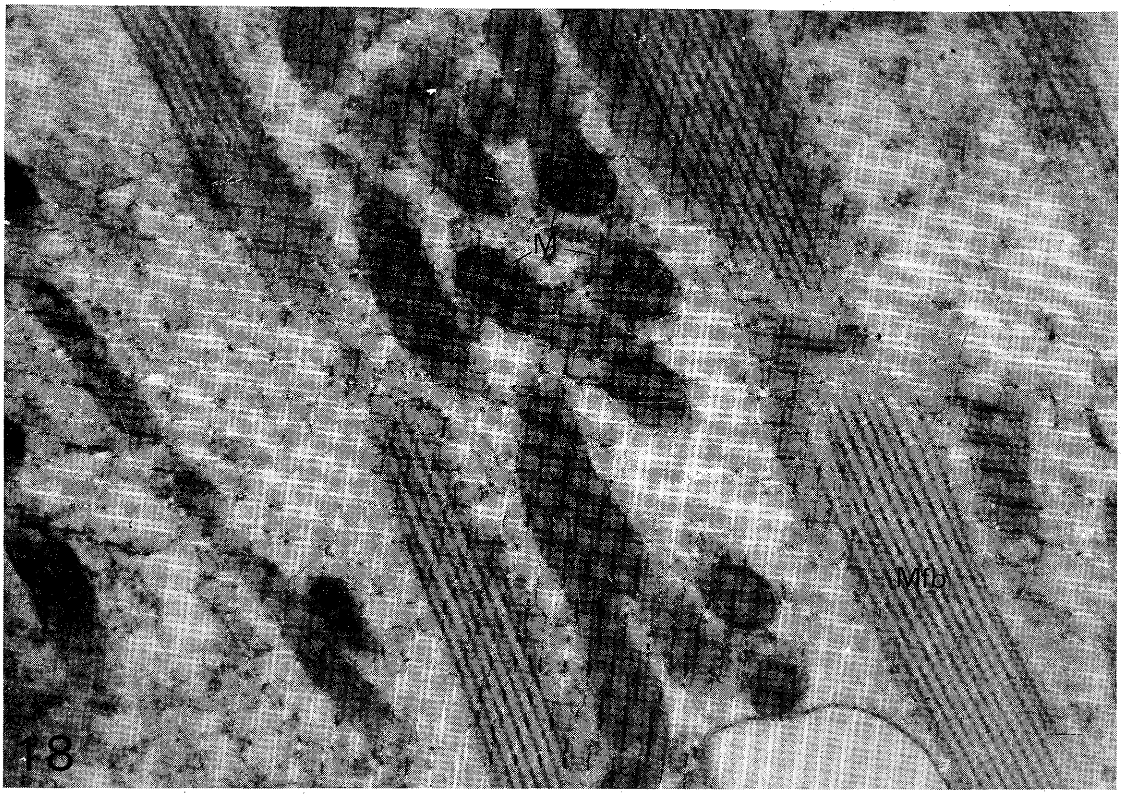
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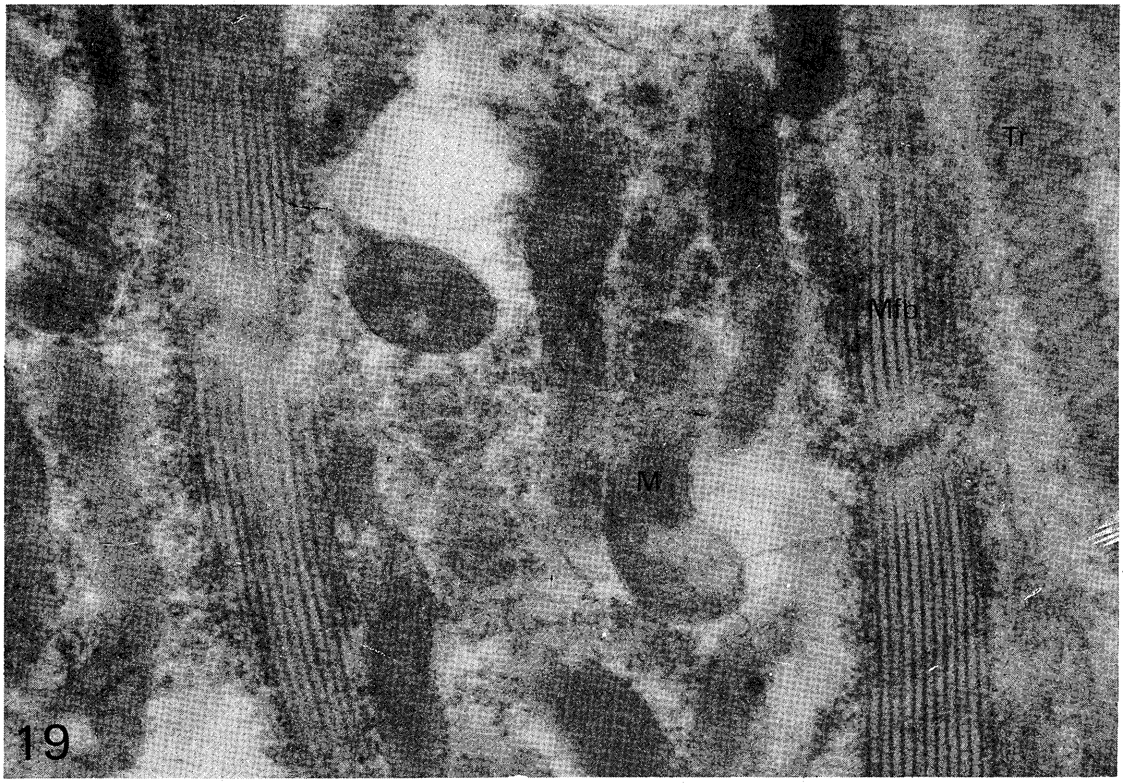
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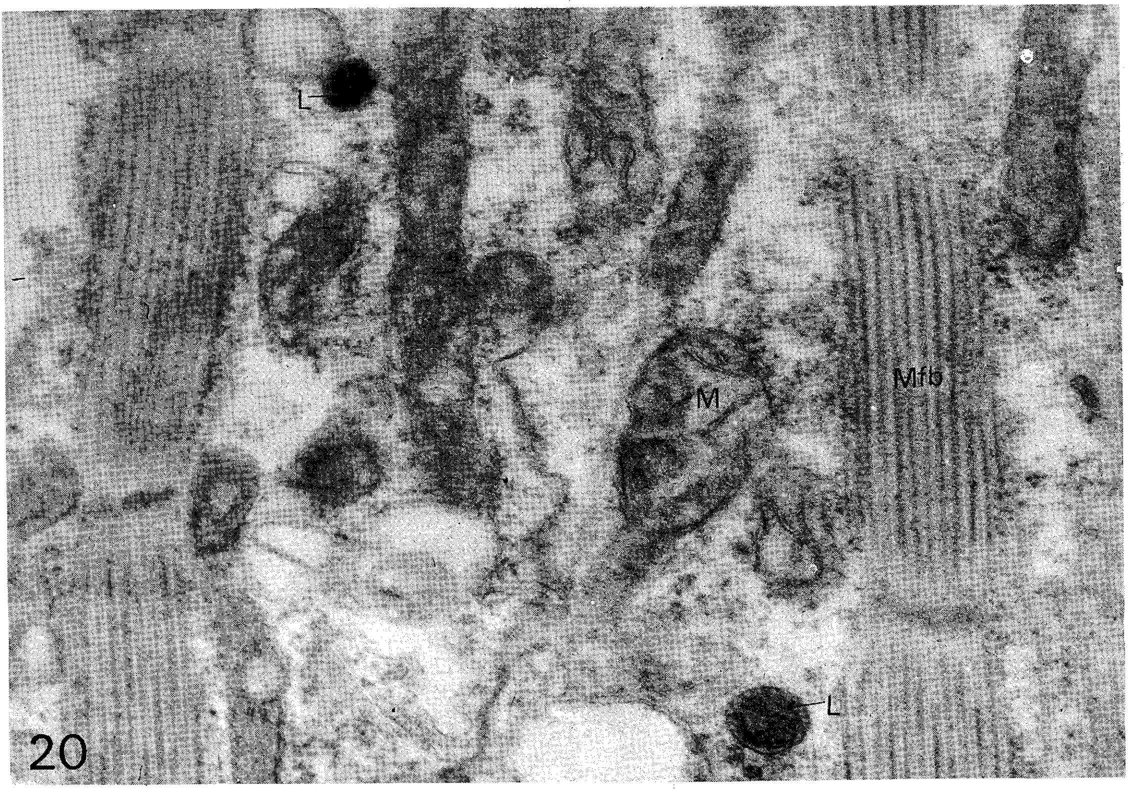
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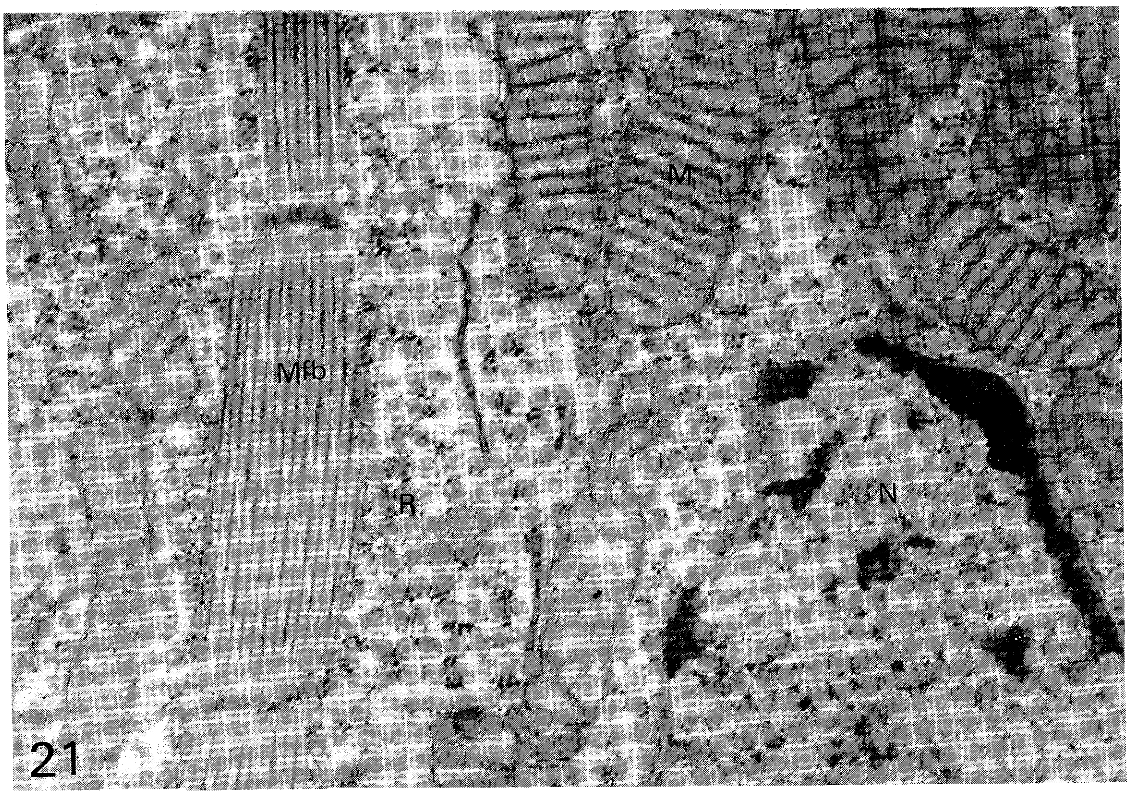
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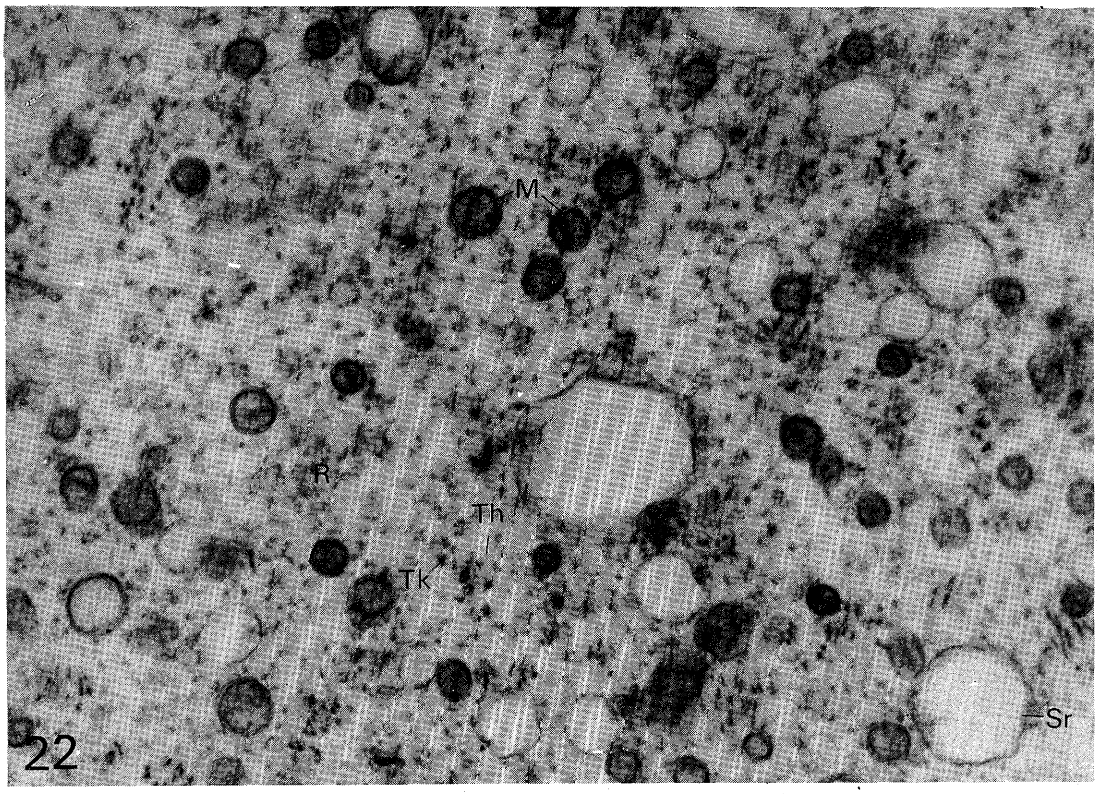
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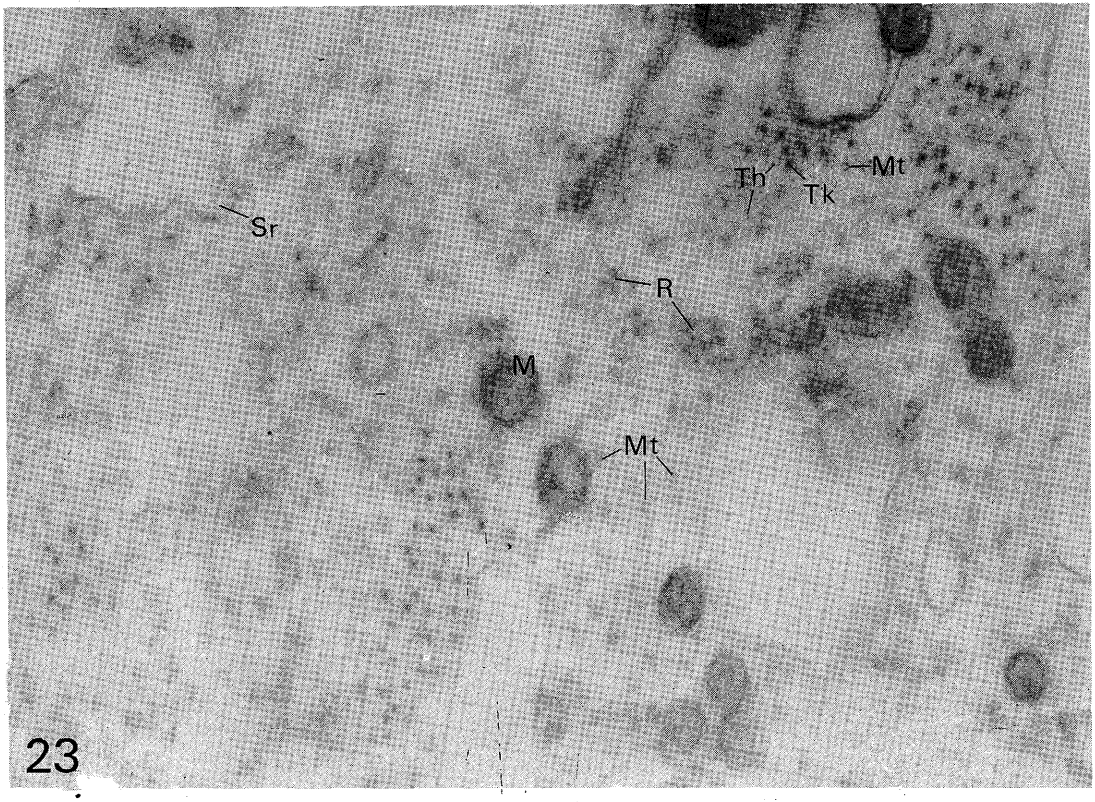
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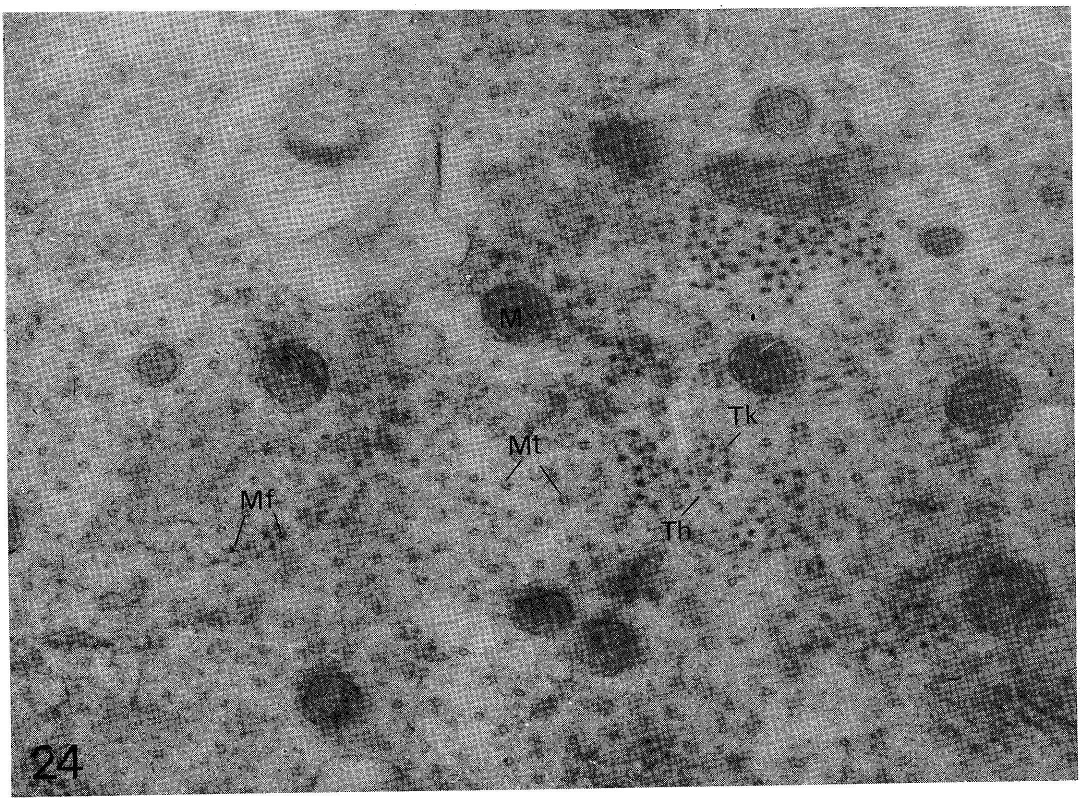

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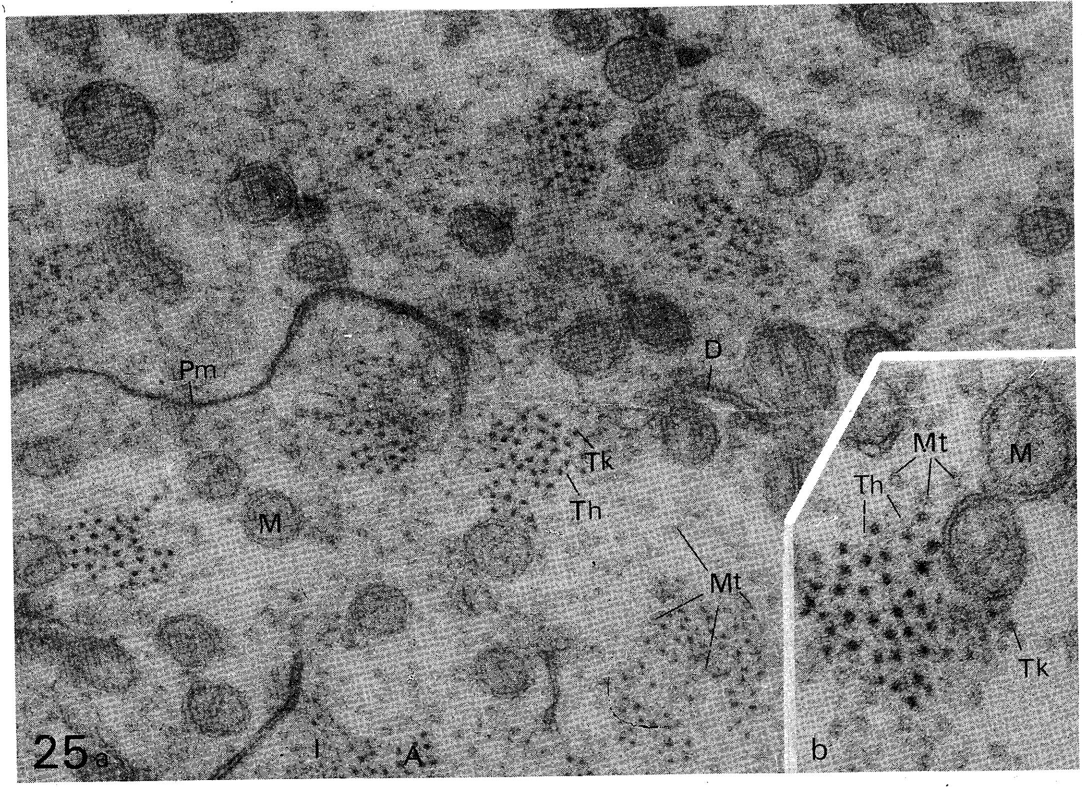

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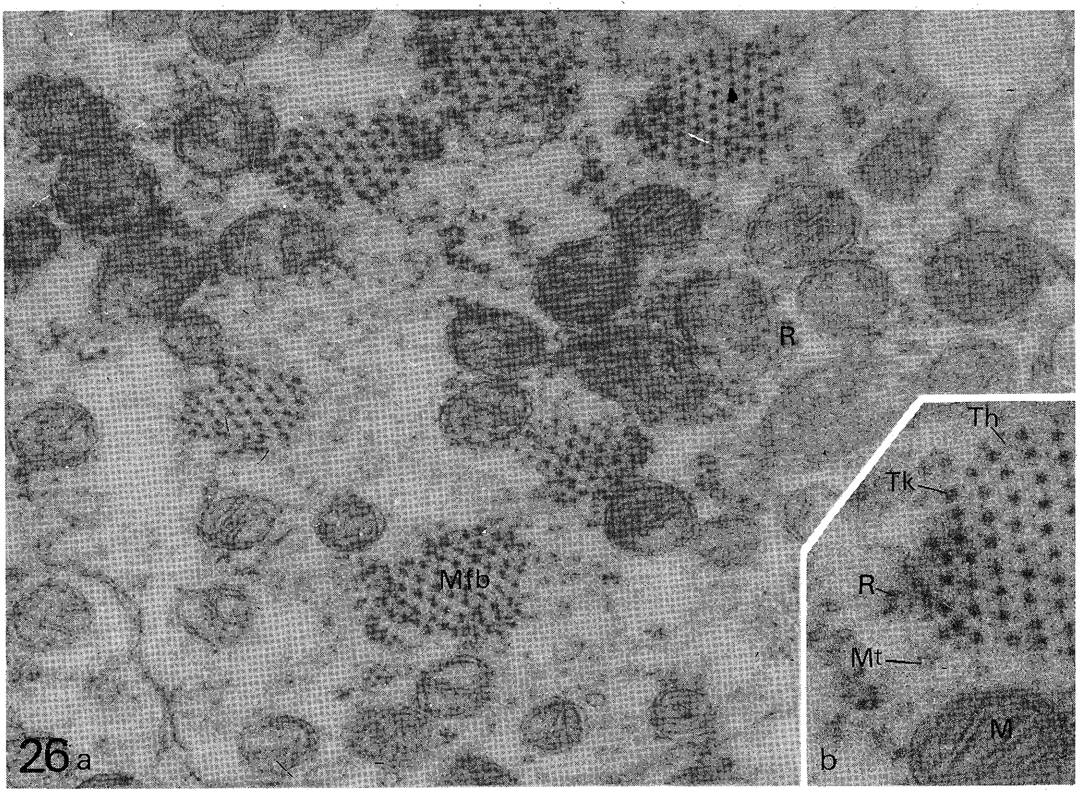
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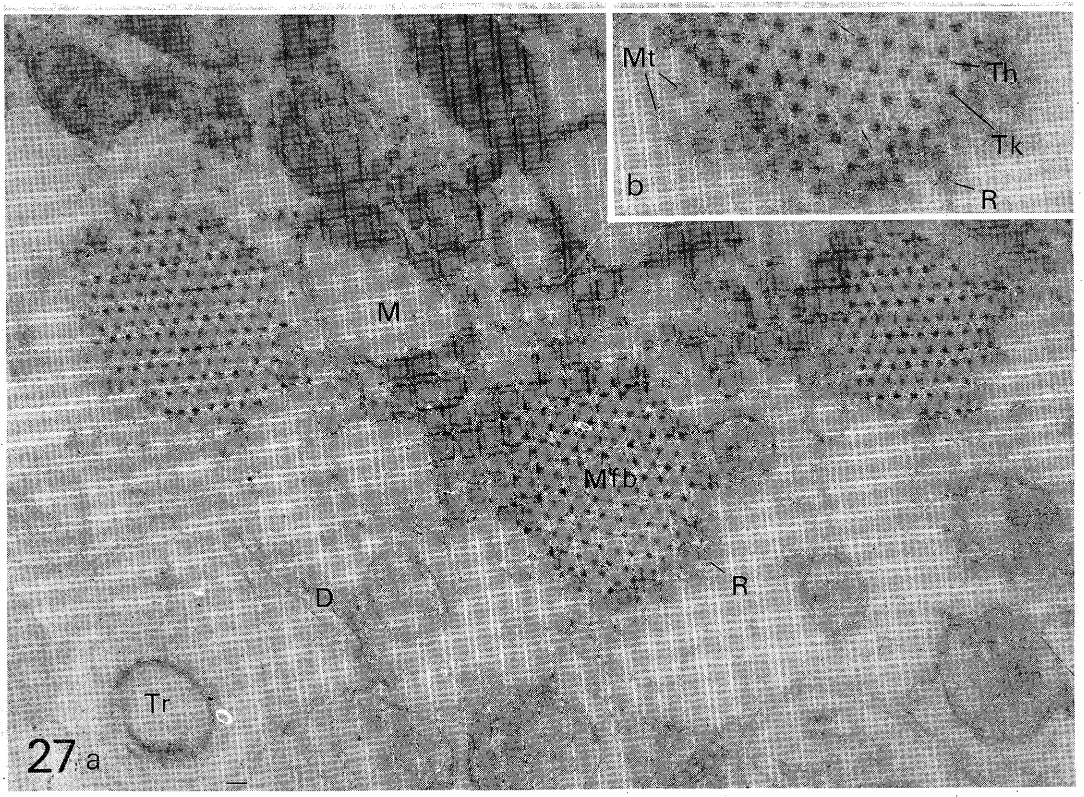
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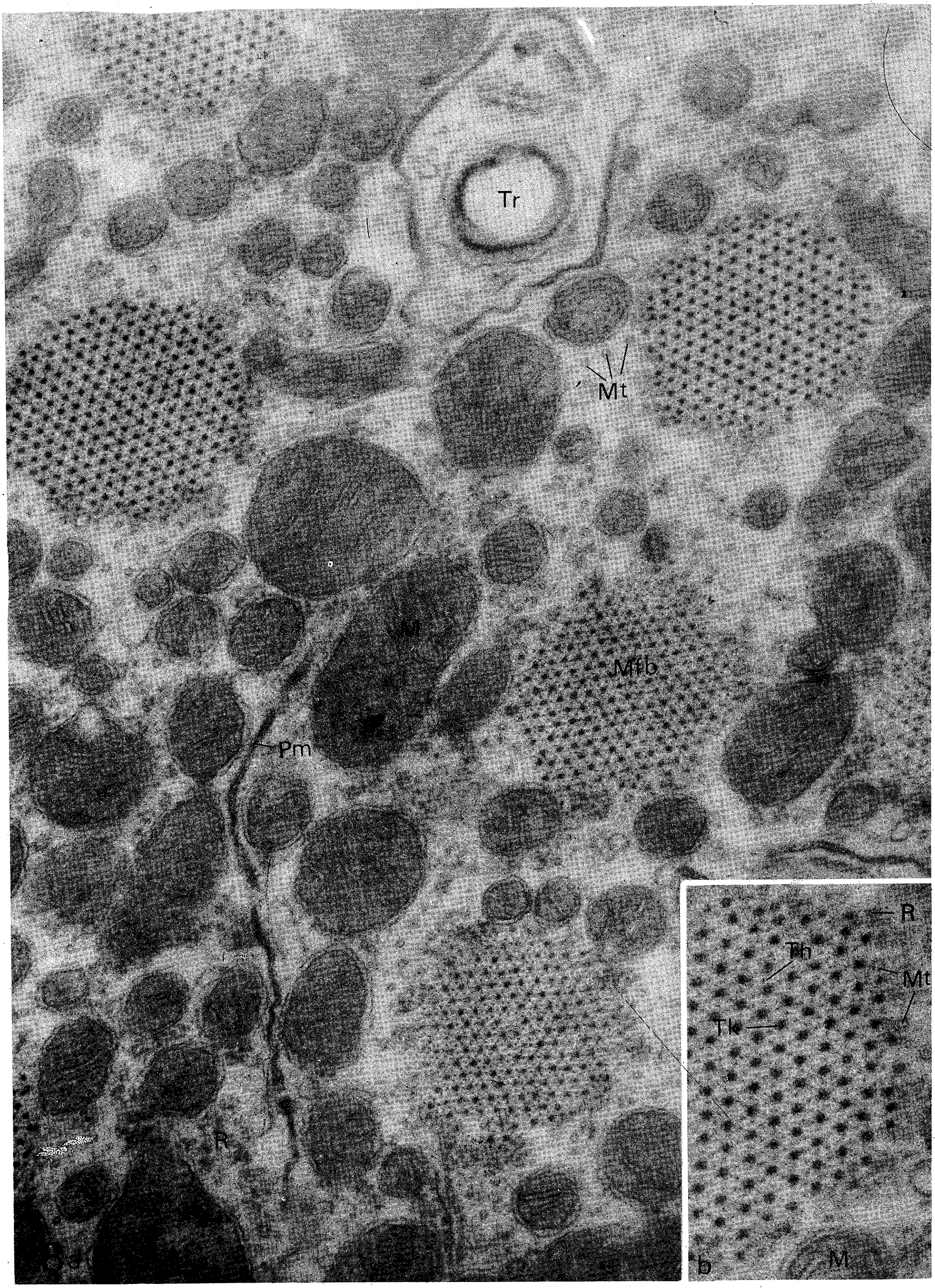
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